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DIVERSITY OF TWO GENES FROM CHEWING PEST *PLUTELLA XYLOSTELLA* (L.) POPULATIONS NATIVE TO DIFFERENT GEOGRAPHICAL REGIONS OF KARNATAKA, INDIA

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ABSTRACT

Plutella xylostella (L.), the Diamondback Moth (DBM), is a serious pest of cruciferous crops across the world, resulting in crop damage. To evaluate the extent of genetic variation in this species, five populations were collected across Karnataka, India. Two important genes: Ecdysteriod receptor(EcR) and Juvenile hormone epoxidase hydrolase (JHEH) were considered for the same. The former is involved in molting process wherein, the later in reproduction and development. In the present study, we have successfully cloned and sequenced both the genes which resulted in 671 and 798 bp amplicons for both EcR and JHEH gene respectively. Further, on sequence alignment with Bioedit, neither nucleotide variation nor parsimony regions for EcR were reported. In case of JHEH, 56 (7.01 %) nucleotide variations along with 43 parsimony informative sites were chronicled. Further, translating nucleotides, 223 and 266 amino acids were generated respectively. Phylogenetic analysis supported that, populations of DBM under study clustered into a single clade, deciphering perseverance of low genetic variations. The data generated with this study aids in designing a safe and effective management strategy like Ribonucleic acid interference (RNAi).

KEYWORDS: Ecdysteriod Receptor, Juvenile Hormone Epoxidase Hydrolase, RNAi, Genetic Diversity

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Original Article

INTRODUCTION

Amidst various Lepidopteran insect pests affecting cruciferous crop like cabbage (*Brassica oleracea*) across the world, the Diamondback Moth (DBM), *Plutella xylostella* (L.) is paramount (Lim, 1986). Crop damages caused due to DBM infestation may even reach 100 % (Calderon et al., 1986). Global annual management expenditure costs one billion US dollars for DBM alone (Talekar et al., 1993; Capinera, 2001; Ahamad et al., 2012). Present management strategies rely upon the overuse of chemical insecticides including organophosphates, Pyrethroids, avermectins, pyrazoles and oxadiazines, which has resulted in resistance gain (Liu et al., 2012, Zhao et al., 2006, Santos et al., 2011). Also, DBM is the first insect pest that has developed resistance against *Bacillus thuringiensis* protein toxins at field levels (Sarfraz et al., 2005).

At this juncture, a safe, eco-friendly and efficient management strategy is required to control the outburst of this insect pest. Hence, understanding the genome for better knowledge on intraspecific variations could be a resort in confirming population structure and dynamics, their adaptability and response to different selection pressures, which aid in developing efficient biological pest management system like Ribonucleic acid interference (RNAi). Since RNAi exhibits sequence precision in degrading the target mRNA, it can be used as bio-pesticide

(Caplen et al., 2000; Baum et al., 2007; Price et al., 2008; Whyard et al., 2009; Mao et al., 2007) against chewing insect pest like DBM.

The aim of this study was to enumerate the genetic variations of two vital genes such as Ecdysteriod receptor (EcR) and Juvenile hormone epoxidase hydrolase (JHEH) from DBM population native to different geographical regions of Karnataka, India. The former belongs to nuclear receptor family functioning in molting process wherein the later, a multi-functional gene that aids development and reproduction in insects.

MATERIALS AND METHODS

Insect Collection

A field population of DBM (Larvae) were collected into RNA stabilizing solution (RNA later ®-Ambion, USA) across cabbage growing regions of Karnataka, India (Table 1). Specimens were stored at -80º C until further molecular studies.

RNA Extraction and Gene Specific Primer Designing

Total RNA was extracted from single DBM specimen by MACHEREY-NAGAL GmbH kit (Germany) as per manufacturer's protocol. Quantification of total RNA was carried out in NanoDrop Lite (Thermo Scientific, Germany) and stored at -20º C until further use. Prior to cDNA synthesis, traces of DNA contamination was removed by treating the RNA with RNase free DNase-I (Fermentas Life Sciences, Germany). Gene specific primers were synthesised with the aid of online tool (Integrated DNA technologies) by retrieving nucleotide sequences from NCBI-GenBank specific to EcR and JHEH for DBM.

cDNA Synthesis and Candidate Gene Isolation

First strand cDNA synthesis was carried out using RevertAid First Strand cDNA synthesis kit (Fermentas Life Sciences, Germany) as: 1.0 µg of RNA was added with 0.5 µg/µl of Oligo (dT) 18 primer and incubated at 65ºC for 5 min. Later, 50 mM EDTA was added to the same vial and incubated at 65ºC for 10 min. The vial was chilled on ice. Later a reaction mixture comprising of 5X reaction buffer, RiboLock RNase Inhibitor (20 U/µL), 10 mM dNTP mix, RevertAid M-MuLV RT (200 U/µL) was added to the same ice chilled vial. Finally the reaction volume was make up to 20.0 µl. The vial was briefly centrifuged and incubated at 42ºC for 60 min. RT activity was terminated by increasing the temperature to 70ºC for 5 min. Quantification of cDNA was carried out by NanoDrop Lite (Thermo Scientific, Germany).

Coding sequences were amplified using gene specific primer (Table 2) from first strand cDNA as follows: 10x reaction buffer; 10 mM each of dATP, dTTP, dGTP and dCTP; 10pM of both sense and anti-sense primers (PxEcR and PxJHEH) for both the genes respectively; 1U of Taq DNA polymerase (TaKaRa, Canada); (1:3) diluted cDNA. Finally, the reaction volume was made up to 50.0 µl using molecular biology grade water (Sigma-Aldrich). Invitro amplification (PCR) was carried out in Veriti 96 wells thermal cycler (AB-Applied Biosystems, USA) with the thermal conditions as: 94 ºC for 4 min (initial denaturation) followed with 35 cycles for 40 Sec at 94 ºC (denaturation); annealing for 40 Sec (51 ºC and 56 ºC for EcR and JHEH respectively); extension for 45 Sec at 72 ºC and ended with 72 ºC for 10 min as final extension. The amplicons were separated on 1.0 % agarose gel stained with ethidium bromide (10 µg/µl) and visualised under UV-trans-illuminator (Bio-Rad, UK) for conforming the molecular size of the amplified product and documented.

Molecular Cloning and Sequencing of Candidate Genes

In compliance with the desired product size, the gel was excised and purified using Nucleospin extract II kit (MN, Germany) as per the standard protocol. Ligation of the eluted product was carried out using a general purpose cloning vector (PTZ 57R/T) (Fermentas, GmbH, Germany) and cloned into *Escherichia coli* (DH5α-strain) as per manufacturer protocol. Blue-white colonies screening was carried out in selecting the positive clones. Further, plasmid isolation was carried out from the overnight inoculated positive clones maintained in LB broth. Using universal M13 primers, sequencing was carried out both in sense and antisense directions using an automated sequencer (ABI Prism® 3730 XL DNA Analyser) (Xcelris Labs, Ahmadabad, India).

Divergence Analysis

Using NCBI-BLAST (http://WWW. ncbi.nlm.nih.gov), homology search for the candidate gene was carried out. Further employing sequence alignment tools such as Bioedit (V.7.0.9.0) (Hall, 1999) and MEGA 6.0 (Tamura et al., 2013), the gene sequences were aligned for evaluating variations. Gene sequences generated in the present study were deposited in NCBI-GenBank (Table 1). With the aid of K2P (Kimura-2-Parameter), Neighbour-Joining tree was constructed (Kimura, 1980; Saitou et al., 1987).

RESULTS

The amplicons generated in the present study were 671 and 798 bp for EcR and JHEH gene respectively. We have successfully cloned and sequenced both the genes collected from 5 different populations. NCBI-BLAST analysis revealed that the candidate genes hit the earlier submitted EcR (EF 417852) and JHEH (NM_001305537.1) gene with 95 and 100% homology. On aligning candidate genes with earlier submitted gene sequences by Bioedit (V.7.0.9.0), our coding sequences are positioned at 188-873 and 94-891 base pairs in complete length sequences. Further, on analysis with MEGA 6.0, neither nucleotide variation nor parsimony informative sites were observed in case of EcR. While with JHEH, there were 56 (7.0 %) nucleotide variations out of 798 bp with 25 parsimony informative sites. Further, the nucleotide frequencies observed for both the EcR and JHEH genes were 22.95 % (A), 13.26% (T), 32.04 % (C), 31.74 % (G) and 28.59 % (A), 22.95 % (T), 26.25 % (C), 22.20 % (G) with maximum composite likelihood (MLC) Transition/Transversion bias (R) being 0.466 and 5.92 respectively. Sequentially, 223 and 266 amino acids were generated upon translating nucleotides sequences of EcR and JHEH genes respectively. Alignments of nucleotide sequences were carried using Bioedit (V.7.0.9.0) to evaluate their variation (Figure 3, 4).

Phylogenetic Analysis

A rooted phylogenetic tree was constructed based on nucleotide sequences of both EcR and JHEH genes respectively using MEGA V.6.0 (Tamura, 2013). Neighbor-Joining model was implemented by selecting bootstrap test (1000 replicates) (Felsenstein, 1985). Further, evolutionary distances were computed with the aid of Kimura 2-parameter method (Kimura, 1980) which was found to be 0.1 for both EcR and JHEH genes. Clustering pattern for both the genes in the phylogenetic tree deciphered that all five populations were very closely related and had less genetic variations (Figure 1, 2).

DISCUSSIONS

Candidate gene such as: EcR belongs to a family of nuclear receptor. EcR was first noticed in *Drosophila*

melanogaster (Koelle et al., 1991). EcR is pivotal in controlling growth and development, molting, embryogenesis, and reproduction (Yamanaka et al., 2013; Carney et al., 1997; Li et al., 2000; Baehrecke et al., 1996; Schwedes et al., 2012). Silencing EcR profoundly alters various vital functions like larval-pupal commitment, anti-feeding activities and post-molting deformations, thereby accomplishing pest management.

Another candidate gene, Juvenile hormone epoxidase hydrolase codes for multifunctional enzyme which degrades Juvenile hormone levels during insect development (Khlebodarova et al., 1996; Wojtasek et al., 1996). JHEH is pivotal in JH pathways making Juvenile Hormone form an irreversible non biological compound known as diol on hydration of epoxide moiety at C10-C11 region (Roe et al., 1990; Wojtasek et al., 1995; Anspaugh et al., 2005; Newman et al., 2005). Hence, knockdown of JHEH will increase JH titre in insect. Thus, it disrupts the feedback mechanism and checks the normal growth and reproduction in insects.

Advancements in molecular biology impacted several corners in biology including population genetics. Recording genetic variations across different geographical populations not only affirms a priority in understanding the pest population, but even aids in developing pest management strategies (Kambhampati et al., 1990). It also assesses nature, selection forces and dynamics of population (Nei 1987; Pamilo 1984; Fakrudin et al., 2004). Hence the present study enumerates the nucleotide variations of candidate genes (EcR and JHEH). Further, the study unravels the existence of low genetic variations within population under study. This can be endured by constructing a phylogram which bunched into a single cluster. Documenting genetic variation also facilitates an innovative approach in pest management such as, RNAi, which is a sequence specific degradation mechanism (Dykxhoorn et al., 2003). As per the present study, the RNAi technology can be used for all the population under study, as both the genes have a very low genetic variation.

CONCLUSIONS

In summary, data generated with the present study on genetic diversity of Ecdysteriod receptor (EcR) and Juvenile hormone epoxidase hydrolase (JHEH) from *Plutella xylostella* will be a foot mark towards development of futuristic insect pest management strategies like RNAi.

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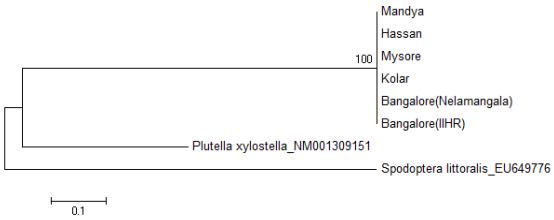
APPENDICES

Table 1: DBM Collection Locations along with NCBI Accession Numbers for Nucleotide Sequences

Gene	Collection Locations	NCBI Accession Numbers
EcR	Bangalore	KT868860
	Kolar	KT868861
	Mysore	KT868862
	Mandya	KT868863
	Hassan	KT868864
JHEH	Bangalore	KT868855
	Kolar	KT868856
	Mysore	KT868857
	Mandya	KT868858
	Hassan	KT868859

Table 2: List of Primers used for *Plutella xylostella* Gene Amplification

Gene	Primer Pair	Primer Sequence (5'-3')	Product Size
ECR	PxEcR	F: CTACGACGACTCCATCACCTACA R: GCCTGGCTATGAGGAACTTCT	671 bp
JHEH	PxJHEH	F:AGGCGATGTCTTAGATACG R: CCCGTACTTGATCAGAGTCT	798 bp



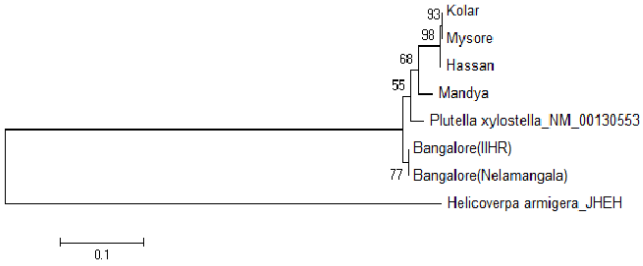


Figure 1 **Figure 2**

Figure 1, 2: Phylogenetic Trees for *P. xylostella* Constructed using MEGA 6.0 Based on Nucleotide Sequences for EcR and JHEH Genes Respectively. The Out Groups Considered are *Spodoptera littoralis* and *Helicoverpa armigera* for EcR and JHEH Respectively

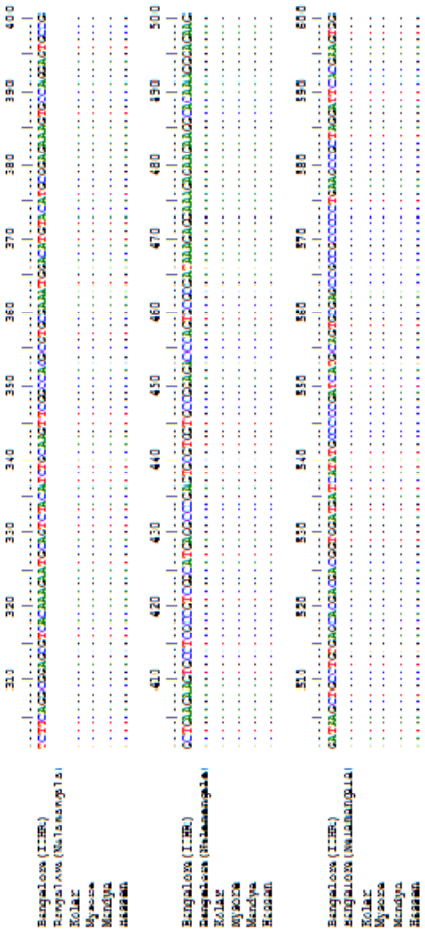


Figure 3: Nucleotide Sequence Allignment of *P.xylostella* EcR Gene Using BIOEDIT

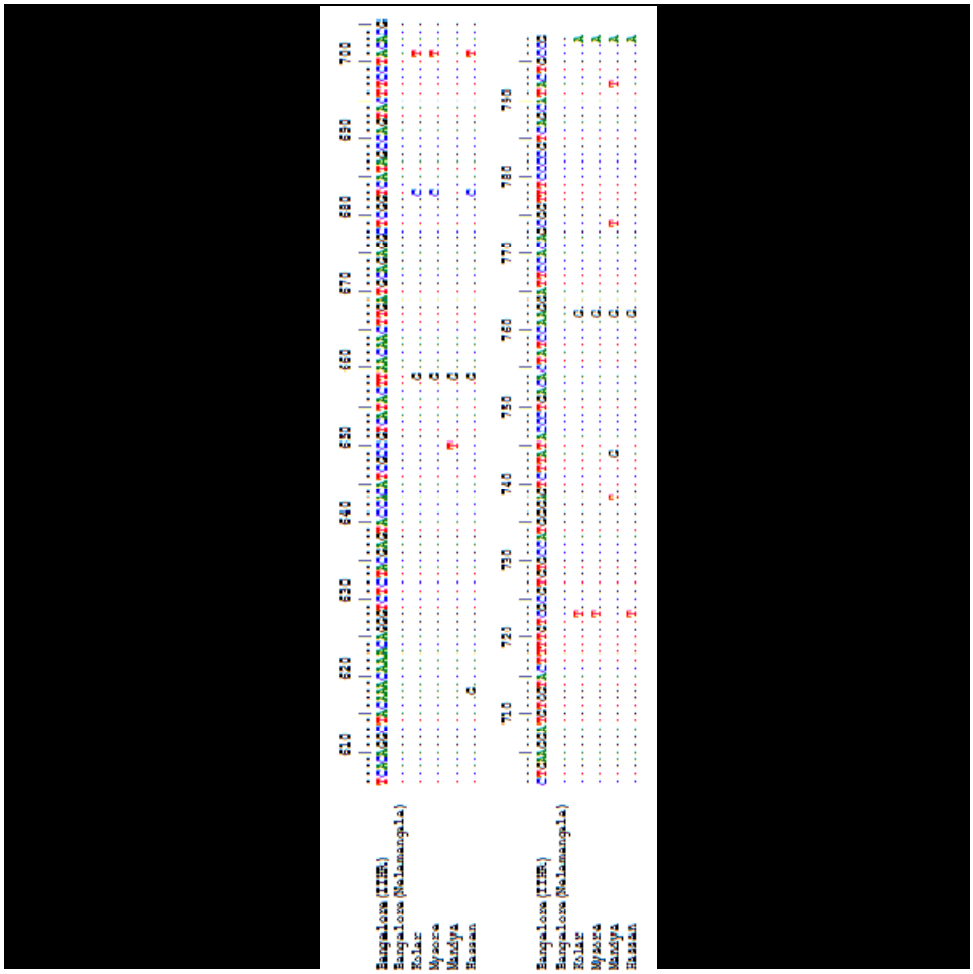


Figure 4: Comparisons of Nucleotide Sequences of *P. xylostella* JHEH Gene Using BIOEDIT